J Mater Sci: Mater Med (2008) 19:2029–2038
DOI 10.1007/s10856-007-3291-3

Long term results after implantation of tissue engineered cartilage for the treatment of osteochondral lesions in a minipig model

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Received: 28 June 2007 / Accepted: 25 September 2007 / Published online: 24 October 2007

Abstract In present study we determined the long term *in vivo* integration and histological modeling of an *in vitro* engineered cartilage construct. Tissue engineered autologous cartilagenous tissue was cultured on calcium phosphate cylinders and implanted into osteochondral defects into the femoral condyles in minipigs. Radiological follow-up was performed at 2, 8, 26 and 52 weeks, condyles were harvested 26 and 52 weeks post-implantation. Thickness of cultivated tissue (1.10 ± 0.55 mm) was comparable to *in situ* cartilage and cells produced *in vitro* cartilage specific proteins. *In vivo*, 26 and 52 weeks post-implantation defects were resurfaced with hyaline-like tissue, the implants were well integrated with no gap at the interface between the engineered neocartilage and the adjacent articular cartilage. Synthesis of type II collagen was detected 26 and 52 weeks after implantation. The modified ICRS score increased from 26 to 52 weeks. Histomorphometric evaluation revealed a decrease in cellularity in tissue engineered cartilage from 2.2-fold of native cartilage after 26 weeks to 1.5-fold after 52 weeks. In conclusion, these findings demonstrate the integration and maturation of tissue engineered cartilage pellets attached on a bone substitute carrier implanted in osteochondral defects over a long time.

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1 Introduction

A variety of therapeutic strategies such as bone marrow stimulation techniques are widely used for the therapy of chondral or osteochondral defects [1, 2]. However, these techniques generate only fibrocartilagenous repair tissue. The transplantation of autologous cartilage-bone plugs and the transplantation of autologous cultured chondrocytes have been advocated as alternative methods [3, 4]. Prospective randomized clinical trials have compared both therapeutic methods, however controversy remains regarding the outcome of these approaches [5–7].

In this regard tissue engineering has attracted great attention because of its potential to promote healing of articular cartilage [8, 9]. Since chondrocytes are harvested from a small biopsy and proliferated and redifferentiated in

cell culture, *in vitro* generated autologous cartilage constructs facilitate the therapy of even larger defects. But this approach is limited by the natural inability to secure the cartilage to bone. To overcome this problem, we covered a microporous biodegradable calcium phosphate carrier by a layer of tissue engineered cartilagenous tissue. As carrier material Calcibon®, consisting of α-TCP, $CaHPO_4$, $CaCO_3$, precipitated hydroxyapatite and Na_2HPO_4 was used. Studies on this combination have demonstrated a good bone biocompatibility as well as a high osteoconductive potency. Calcibon® can be fabricated in different shapes and porosities [10, 11].

Based on the fact that bone will integrate the bone substitute after implantation, biological anchorage of the cartilage should be achieved. The cultivation procedure used in this study is based on high density cultivation of chondrocytes recovered from alginate gel. Incorporation of cell-attached matrix into the constructs resulted in cartilaginous tissue suitable for implantation, omitting artificial scaffolds for the cartilage layer.

The objective of this study was to establish the methodology and to evaluate the long term *in vivo* performance of the *in vitro* generated tissue in a large animal model.

2 Materials and methods

2.1 Animals

The study has been approved by the appropriate governmental review board for animal experimentation. Animals were kept according to FELASA-guidelines. Eight Göttinger minipigs with a mean age of 27 months (range 18–46) and mean weight 40 kg were obtained from Ellegaard Göttingen Minipigs ApS, Dalmose, Denmark and kept under conventional housing-conditions. Quarantine lasted 7 days. The animals were housed with appropriate bedding, provided with free access to drinking water and food. Based on the knowledge in the literature about the healing process of osteochondral defects [12–15], osteochondral control defects were not performed as a request of the Animal Ethics Committee of the state of Hamburg.

2.2 Calcium phosphate carrier

Calcium phosphate carriers consisting of Calcibon® (2 mm in height and 4.5 mm in diameter) were kindly provided by Biomet Merck Biomaterials Darmstadt, Germany. Calcibon® is an injectable, *in situ* setting bone cement [10, 16]. This commercially available material is also referred to in the literature as biocement D [11, 17]). Calcibon® consists of a powder (61% alpha-tri-calcium-phosphate, 26% calcium-hydrogen-phosphate, 10% calcium-carbonate, and 3% precipitated hydroxyapatite) and a liquid component (di-sodium-hydrogen-phosphate). After setting, the material is finally transformed into microcrystalline, carbonated, calcium deficient hydroxyapatite [10]. As described by Oreffo *et al.* 1998 [17], the carriers were prepared by mixing the two components of Calcibon® and by injecting the cement into a circular-shaped mould to obtain the required size and shape of the discs. Before setting, the surface of the bone cement was covered with granula (200–220 μm in size) of Calcibon®. These granula improved cell coating and attachment of cartilage tissue during the cultivation and implantation process. The discs were removed from the moulds after the cement had set and rinsed in 0.9% saline for 16 h. For the cultivation of cartilage tissue, the carriers were sterilized by autoclaving and inserted in tissue culture as described before [18].

2.3 Isolation and expansion of articular chondrocytes

Cartilage biopsies from Göttinger minipigs were obtained under general anesthesia. Chondrocytes were isolated by sequential digestion with hyaluronidase and collagenase (0.5 mg/mL, SIGMA-Aldrich, Taufkirchen, Germany). Cell numbers were determined using a CASY-counter (Schärfe System, Reutlingen, Germany). Cells were grown in Dulbecco's modified Eagles medium (DMEM, Invitrogen, Karlsruhe, Germany) containing 10% fetal bovine serum (PAA Laboratories, Coelbe, Germany), 100 u/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Karlsruhe, Germany) 10 ng/mL bFGF (R&D Systems, Wiesbaden, Germany) and maintained at 37 °C, 5% CO_2.

2.4 Cell coating of calcium phosphate carriers

Two to three passages following biopsy, adherent cells were released from the dishes by exposure to 0.25% trypsin/EDTA. Chondrocytes ($2–4 \times 10^5$) were seeded on the calcium phosphate carriers in order to improve adherence of cartilagenous tissue produced later on. Cell seeded carriers were then cultivated for two weeks in DMEM containing 10% fetal bovine serum and bFGF (10 ng/mL).

2.5 Cultivation of expanded chondrocytes in alginate

Expanded chondrocytes were encapsulated in alginate gel and cultivated for two weeks as described by Yaeger et al. [19] in order to promote redifferentiation and formation of cell associated matrix. 3×10^7 cells at a density of 1×10^6/mL were mixed at room temperature in 1.2%

buffered alginate (25 mM HEPES pH 7.4, 150 mM NaCl, 1.2% sodium alginate from SIGMA-Aldrich, Taufkirchen, Gemany) until a homogeneous suspension was obtained. The suspension was pushed gently through a needle so that drops fell into a solution of calcium chloride (25 mM HEPES pH 7.4, 100 mM $CaCl_2$, VWR). Alginate beads with a diameter of 2 mm, a volume of 50 µl and a cell density of about 5×10^4 formed instantaneously and polymerized after 20 min. Calcium buffer was aspirated and the beads washed twice with washing buffer. Alginate beads were cultivated in DMEM containing 10% porcine serum (Invitrogen, Karlsruhe, Germany), 100 u/mL penicillin, 100 µg/mL streptomycin, ascorbic acid (50 µg/mL), IGF-I (100 ng/mL, R&D Systems, Wiesbaden, Germany) and TGF-ß (10 ng/mL R&D Systems) at 37 °C, 5% CO_2 and 5% pO_2.

2.6 Formation of osteochondral implants

Two weeks later chondrocytes were eluted from the alginate beads by addition of 5 volumes of sodium citrate (55 mM) in washing buffer. Recovered cells were then evaluated by phase contrast microscopy and by visualizing cell associated matrix with DMMB solution. For subsequent tissue cultivation, recovered chondrocytes were washed with washing buffer, resuspended in medium and sedimented (1×10^6 counts, $70 \times g$) on the cell coated calcium phosphate carriers. These biphasic constructs were further cultivated for three weeks in DMEM containing 10% porcine serum, 100 u/mL penicillin, 100 µg/mL streptomycin, ascorbic acid (50 µg/mL), IGF-I (100 ng/mL) and TGF-ß (10 ng/mL).

2.7 Characterization of osteochondral implants

In vitro data were determined as previously described [18]. Ten implants were cultivated for each animal. Two of the constructs were used for implantation, remaining implants were used for biochemical, histological and immunohistological evaluation. After removal of the tissue from the calcium phosphate carriers, wet weight was determined and thickness was measured using a digital micrometer. To determine total GAG and DNA contents of redifferentiated chondrocytes and of osteochondral implants, dimethylmethylene blue (DMMB) and H33258 assays were performed as described by Buschmann et al. after digestion with papain [20]. Prior to analysis, recovered chondrocytes were washed twice in washing buffer to remove remaining alginate. Hydroxyproline content was determined after hydrolysis with 6 M HCl. Histology and immunohistochemistry were performed after decalcification with 6% EDTA. Cell seeding of the calcium phosphate carriers was verified using scanning electron microscopy.

2.8 Generation of osteochondral defects and implantation of biphasic constructs

All operations were performed approximately 50 (47–53) days after biopsy. Both knees were operated in all animals ($n = 8$). Using general anesthesia and sterile technique an anteromedial mini-arthrotomy of 3 cm was performed and the patella was laterally dislocated. A steel drill (4.5 mm in diameter) with a stop-device was used to create a full-thickness defect (3.0 ± 0.5 mm deep) in the weight bearing zone of the medial femoral condyle. The defects were cleaned and rinsed with sterile saline. The autologous biphasic constructs were press-fitted into the drill-holes after the chondral layer was cut on the edges to create a fresh border at the implantation site. The articular surface of the cylinder was observed to be congruent with the circumjacent cartilage. A closure in layers was performed. After extubation, all animals were returned to their cages and were allowed free cage activity and full weight bearing.

2.9 Radiographic and magnetic resonance imaging

Anterior, posterior and lateral radiographs as well as magnetic resonance (MR) imaging were carried out on intact joints. The following sequences were undertaken using a Siemens 1.5 Tesla scanner (Siemens, Erlangen, Germany):

1. T2-weighted 3D-DESS (Double Echo Steady State) sequence in sagittal and coroneal planes;
2. T2-weighted TSE (Turbo Spin Echo) sequence in sagittal planes;
3. T1-weighted 3D Flash sequence in sagittal planes.

2.10 Macroscopic and histological evaluation

Animals were sacrificed 26 (n = 4) and 52 weeks (n = 4) respectively after implantation and both knee joints were disarticulated. The site of the biphasic construct implantation was photographed. For paraffin embedding, specimens were dissected out, fixed in 4% formalaldehyde and decalcified in 10% EDTA for 4 weeks, embedded in paraffin, cut transversely at 5 µm sections and stained with either toluidine or hematoxylin/eosin. For methylmethacrylate embedding medial femoral

condyles were sectioned in the center of the sagittal plane using a diamond-coated precision band saw (Exakt, Norderstedt, Germany). Afterwards a 4 mm thick central slice was rinsed and cleaned from marrow, embedded in plastic (methylmethacrylate, Kulzer, Germany), and ground to a thickness of 100 µm. The surface was stained using a modification of the von Kossa method to permit 2- and 3-dimensional analysis as previously described [21]. All specimens were evaluated on light microscopy in terms of cellularity, nature of cells, inflammatory signs, presence or absence of new collagenous matrix as well as of presence of recently synthesized proteoglycans. Cellularity was counted in four defined squares (each 0.1075 mm^2 in size) in the area of native and in four squares in the area of implanted cartilage on every specimen using a Zeiss Axioscope II (Zeiss, Göttingen, Germany). Histological analysis was performed by two blinded observers according to a modified Score of the International Cartilage Repair Society (ICRS) [22]. Criteria V and IV of the score were excluded because the subchondral bone was replaced by a calcium-phosphate carrier and a special staining for cartilage mineralization was not conducted.

2.11 Immunohistochemistry

Immunostaining was carried out using monoclonal antibodies against type I and type II collagen (Clones I-8H5 and II-4C11, Medicorp Inc. Montreal, Kanada). Prior to immunolabelling, sections were enzymatically digested with 0.1 % hyaluronidase III (Sigma) in PBS pH 6.5 for 4 h at 37 °C to unmask collagen epitopes. Negative controls were carried out omitting primary antibodies. Biotinylated goat anti-mouse secondary antibody was purchased from Biozol, Eching, Germany. Labelling was visualized with streptavidin/alkaline phosphatase complex using the New Fuchsin chromogen (Sigma).

2.12 Statistical analysis

All data are given as mean value ± standard deviation. Differences between the groups in Table 2 (cellularity in implants compared to native cartilage) were evaluated by the Tukey test, differences between the groups in Table 3 (ICRS-score) were evaluated by the Mann–Whitney rank sum test. All tests were calculated with a significance level of $\alpha = 0.05$. *P* values less than 0.05 were considered significant. Statistical analysis was performed with SigmaStat (version 2.03) by SPSS®.

3 Results

3.1 Characterization of osteochondral implants

In vitro data of osteochondral implants were determined as previously described [18]. Investigated parameters were physical properties (height and weight), biochemical analysis (glycosaminoglycan, DNA, hydroxyproline), immunoblotting and histological as well as immunohistological evaluation. Isolated cells from cartilage kept their characteristic polygonal morphology until passage two. Cell viability after culture in alginate gel amounted to 92%. After cultivation in alginate gel, cells showed a characteristic halo consisting of pericellular matrix indicating their redifferentiation.

Chondrocytes sedimented on calcium phosphate cylinders formed *in vitro* a continuous cartilagenous layer which achieved with 1.10 ± 0.55 mm a similar thickness to native articular cartilage of minipigs (Fig. 1a and b). The interface between cartilagenous tissue and the carrier was

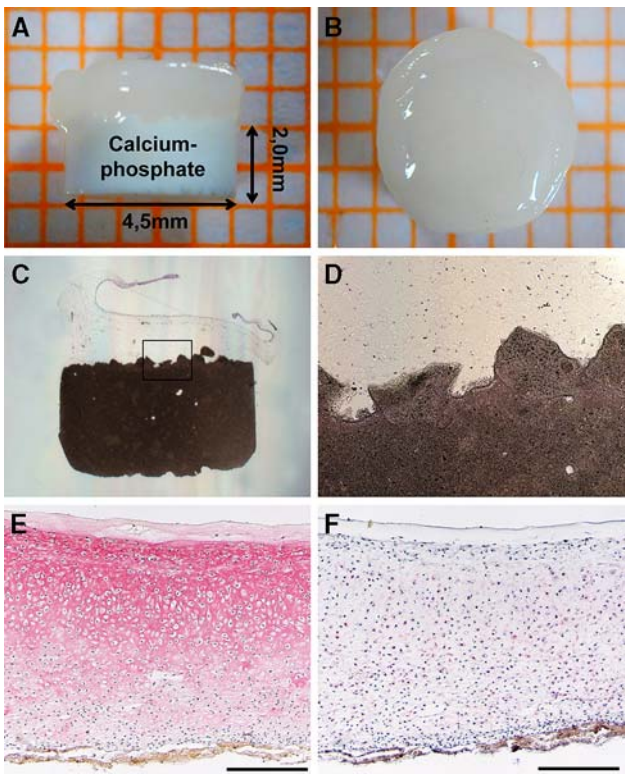


Fig. 1 *In vitro* generation of biphasic constructs. (a, b) Chondrocytes sedimented on calcium phosphate carriers formed a cartilagenous tissue of about 1.10 ± 0.55 mm thickness. (c) Histological examination revealed well distributed viable chondrocytes surrounded by accumulated extracellular matrix (staining Toluidin, original magnification ×40. (d) Detail of the interface in higher magnification demonstrating a well integrated cartilagenous tissue on the calcium phosphate carrier with no visible gap (staining Toluidin, original magnification ×100). (e) and (f) Immunohistological staining of cartilage layer for collagen type II (f) and negative control (f)

Table 1 *In vitro* evaluation of cartilagenous implants

Physical and biochemical properties of tissue	
Thickness (mm)	1.10 ± 0.55
Wet weight of tissue (mg)	35.17 ± 15.35
DNA (μg)*	18.72 ± 10.33 / 23.76 ± 11.67
DNA (μg/mg wet weight)	0.78 ± 0.47
GAG (μg)*	105.15 ± 66.96 / 169.46 ± 78.82
GAG (μg/mg wetweight)	5.29 ± 3.02
Hydroxyproline (μg)	49.33 ± 21.09
Hydroxyproline (μg/mg wet weight)	1.83 ± 1.75

Thickness and wet weight of the tissue were determined after removal from the calcium phosphate carriers. DNA and GAG content were measured after digestion with papain. Values of DNA and GAG-content are given for recovered chondrocytes from alginate gel (*) and for implants. Samples of recovered chondrocytes contained 1×10^6 colonies corresponding to $3.12 \pm 1.72 \times 10^6$ cells. Hydroxyproline content was quantified after hydrolysis of Papain digests with HCl.

in all constructs stable enough to be handled with surgical forceps. Histological examination of the tissue revealed well distributed viable chondrocytes within accumulated extracellular matrix. A good junction and integration of the neocartilage with no visible gap at the interface to the calcium phosphate carrier was observed (Fig. 1c and d).

Immunohistochemistry of the tissue indicated the presence of collagen type II and collagen type I (Fig. 1e and f). Biochemical analysis of redifferentiated chondrocytes revealed the presence of glycosaminoglycans in the cell associated matrix. Glycosaminoglycan (GAG) content was 105.15 ± 66.96 μg per 1×10^6 colonies which were used to build up the cartilage layer on top of the calcium phosphate cylinders (Table 1). During alginate culture, some growth of chondrocytes had occurred. Determination of DNA content of recovered chondrocytes indicated, that cell colonies contained average numbers of $3.12 \pm 1.72 \times 10^6$ cells per 1×10^6 counts assuming 6.05 pg DNA per cell.

Wet weight of tissue layer was 35.17 ± 15.25 mg. GAG content increased to 169.46 ± 78.82 μg and hydroxyproline content of 49.33 ± 21.09 μg per construct was determined. Total cellularity within the cartilagenous tissue amounted to $3.86 \pm 1.90 \times 10^6$ as measured by DNA analysis .

3.2 Radiographic and magnetic resonance imaging

Radiographic follow-up evaluation demonstrated the osteochondral cylinders to be congruent and to be well integrated in the circumjacent tissue in the medial femoral condyle (Fig. 2a). On magnetic resonance (MR) imaging, the thickness of the implanted cartilagenous tissue appeared the same as that of native cartilage. A transient edema at the implant surface and between the calcium phosphate carrier and the adjacent bone which resolved by 26 weeks was observed. Some of the biphasic constructs were countersunk, in consequence the depth of the hole became slightly greater than the initially drilled 3 ± 0.5 mm (Fig. 2b).

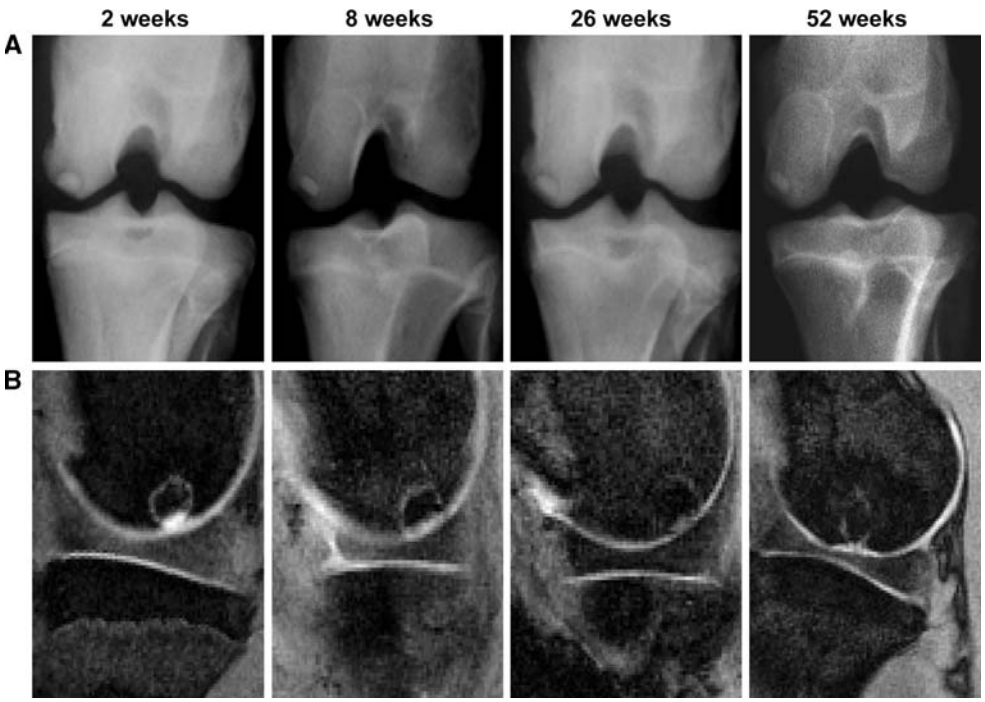


Fig. 2 Radiologic and MR imaging. (a) Anteroposterior radiographs and (b) sagittal T2-weighted 3D-DESS images taken at 2, 8, 26 and 52 weeks after implantation revealed the biphasic constructs to be well integrated and congruent with the circumjacent tissue. An interface between biphasic constructs and the recipient site is even 52 weeks post-implantation definable. The calcium phosphate carrier have not been degraded at this timepoint

3.3 Macroscopic findings

All animals recovered from both operations. One subcutaneous infection occurred without involvement of the joint. The infection was treated by incision and local antiseptic ointment and healed after few days. At final evaluation prior to sacrifies, all animals had completely healed scars and showed normal agility and gait. All knees joints were stable to anterior, posterior, varus and valgus testing and had a normal range of motion. No differences could be detected between the animals of different groups. Gross observation revealed no significant degenerative changes in the medial or lateral compartments of the knees of any of the minipigs. No effusion nor inflammatory signs were observed. The biphasic constructs appeared as they did at time of implantation, the margins of the implants were still visible by gross examination without the appearance of a gap between implant and native articular surface. By touching the implants surface it appeared smoother but as elastic as the surrounding native cartilage, especially in the center of the implant. The donor site demonstrated neither signs of inflammation nor necrosis, but also no signs of cartilage repair. No scarification or chondral lesions were seen on the opposing patella.

3.4 Histological and immunohistochemical findings

Histological examination revealed the biphasic constructs well integrated into the adjacent cartilage and bone (Fig. 3a). Some of the implants were countersunk into the surrounding bone. New bone was grown from the sides of the implants overlapping the underlying calcium phosphate accordant a partial reconstruction of the subchondral bone. After 52 weeks around half of the cylinder surface was covered with bone. No remodeling of the calcium phosphate could be found, the carriers were neither resorbed nor degraded (Fig. 3a). All specimens showed neocartilage–cartilage healing with no gap between the implants and the circumjacent cartilage (Fig. 3b). Chondrocytes appeared viable and within lacunae. Accumulated extracellular matrix could be determined surrounding the cells (Fig. 3b). In some areas, especially on the edges of the chondral part of the implants, fibrocartilage and a mixture of hyaline and fibrous tissue could be determined. Staining with toluidine blue indicated sulfated proteoglycans within the extracellular matrix (Fig. 3a and b). The cartilagenous tissue was integrated with the subsurface region of the bone substitute as it had filled the superficial pores of the calcium phospate. No subchondral cysts were found in the specimens.

The *in vitro* formed cartilagenous tissue appeared more cellular than native cartilage, albeit cellularity decreased from 26 to 52 weeks after implantation. Cellularity amounted in native cartilage 44.5 ± 6.23, in implanted cartilage after 26 weeks 96.0 ± 3.75 and after 52 weeks 66.7 ± 8.04 cells per 0.1075 mm^2. Compared to native hyaline cartilage, the cartilagenous tissue demonstrated 2.2-fold more cells after 26 weeks. After 52 weeks,

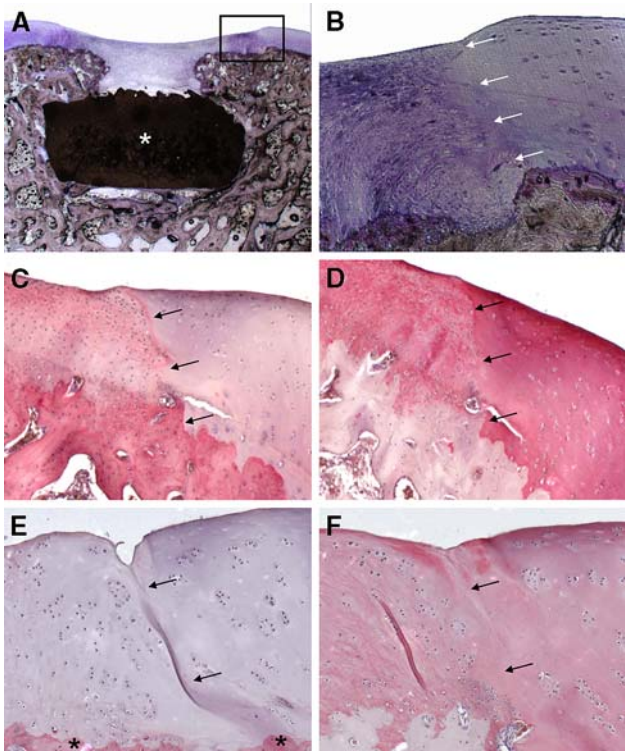
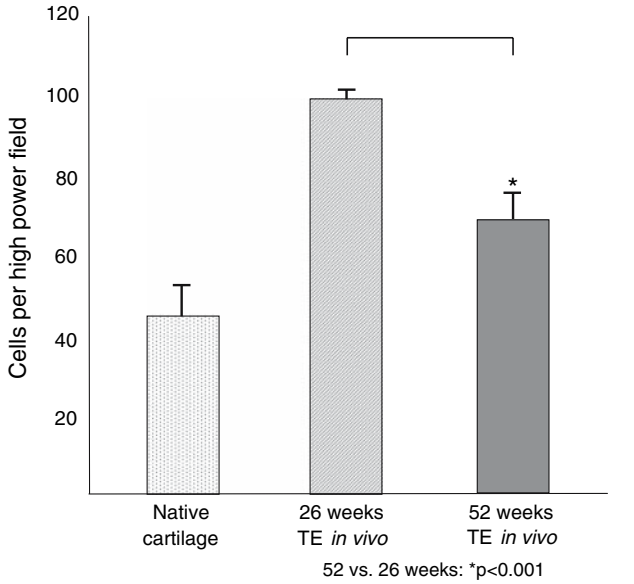


Fig. 3 (a) Histologic sections of femoral condyles 52 weeks after implantation of biphasic constructs revealed a continuous layer of cartilagenous tissue on the surface of the calcium phosphate carrier and a repair of the osteochondral defect by the implant. The biphasic constructs surface was slightly curved. New bone was grown from the sides and overlapped the underlying calcium phosphate meaning a partial reconstruction of the subchondral bone. No resorption nor degradation of the carrier occurred (asterix). (b) Detail of the junctional zone in higher magnification demonstrating the implant to be well integrated with no gap between the engineered and adjacent original cartilage (arrows). In some specimens fibrous tissue in the interface between the calcium phosphate carrier and adjacent bone was apparent (staining toluidine, original magnification (a) ×40, (b) ×200). Sections from the immunostained implants for (c) type I collagen and (d) type II collagen. (c) Type I collagen was seen in all samples but was more discrete and variable than type II collagen. The distribution of type I collagen was predominantly in the very lateral region. (d) Type II collagen was seen throughout most hyaline-like repair tissue similar to that seen in the normal articular cartilage. Note the lack of staining for type II collagen in the bone (original magnification (c) and (d) ×200). (e) High magnification view of the cartilage interface shows the lack of type I collagen signal in the native and only a slight signal in the tissue engineered cartilage while the subchondral bony lamella clearly stains positive for type I collagen (asterix). (f) In contrast type II collagen is found in native as well as tissue engineered cartilage while it is absent in the subchondral bony lamella (original magnification (e) and (f) ×400). In all illustrations implants are located left to the arrows

Table 2 Mean values of chondral cellularity 26 and 52 weeks after implantation of biphasic constructs compared to the native hyaline cartilage



	Native cartilage	26 weeks	52 weeks
Cells per high power field (0.1075 mm²)	44.5 ± 6.23	96.9 ± 3.75	66.7 ± 8.04
X-fold cells of native cartilage	1.0	2.2	1.5

The *in vitro* formed cartilagenous tissue appeared 2.2-fold and 1.5-fold, respectively more cellular than native cartilage. Cellularity decreased from 26 to 52 weeks after implantation, indicating a progressive tissue maturation *in situ*. Statistical significant difference ($p < 0.001$) was determined by the Tukey test in multiple comparison procedures.

cellularity has significantly decreased to 1.5-fold more ($p < 0.001$) indicating a maturation of the tissue engineered construct *in vivo* (Table 2).

Collagen-synthesis was revealed by immunohistochemistry. Staining for type II collagen was positive in all specimens (Fig. 3d and f). Type I collagen immunostaining was seen in all samples but was more variable than for type II collagen (Fig. 3c and e) and more discrete in the 52 weeks group. In most of the implants the staining was discrete and usually restricted to the lateral region.

3.5 Semiquantitative scoring by the modified ICRS score

The semiquantitative scoring after 26 weeks demonstrated in two of the four repair tissues a total score of 7 out of 12 points meaning 58% of the characteristics of normal, hyaline cartilage. One implant reached 83 % (10/12) of the characteristics and only one, in which the surface was irregular and the cells were in a disorganized distribution, reached a score under 50 % (33%, 4/12).

After 52 weeks the average score increased compared to the 26 weeks group (7 to 9). Increase was not significant (p = Two of the implants displayed 10 out of 12 points (83%). These implants showed a smooth, continuous surface. The matrix consist of a mixture of hyaline and fibrocartilage with viable cells in a columnar distribution. Total score values are summarized in Table 3.

4 Discussion

Tissue engineering has long been investigated to repair articular cartilage defects. Technical difficulties in fixing tissue grafts or isolated cell suspension into the cartilage defects have led to experimentation with biodegradable scaffolds. Successful reports have usually involved the seeding of autologous chondrocytes into polymers. However, problems occurred such as acidic product accumulation and side effects in local or systemic inflammatory reactions during *in vivo* degradation [23].

Table 3 Four individual parameters are listed according to the modified ICRS-Score [22]

Category	Range	26 weeks				52 weeks			
		A	B	C	D	E	F	G	H
I	0–3	3	3	0	3	3	3	3	3
II	0–3	1	1	1	2	2	2	2	1
III	0–3	0	0	0	2	1	2	2	0
IV	0–3	3	3	3	3	3	3	3	3
Total	0–12	7	7	4	10	9	10	10	7
Mean ± SD		7 ± 2.1				10 ± 1.2*			

Modified ICRS score: I = Surface, II = Matrix, III = Cell distribution, IV = Cell population viability. Group A–D = 26 weeks after implantation, group E–H = 52 weeks after implantation. The average score of 10 ± 1.2 was higher at 52 weeks post-implantation compared to 7 ± 2.1 at 26 weeks, but no significant difference was detected (Mann–Whitney Rank Sum test, $p = 0.343$).

52 vs. 26 weeks: *$p = 0.343$

Tissue engineering of cartilage was also hampered by the inability of the developed tissue construct to integrate with subchondral bone or the adjacent native cartilage after implantation. To overcome the problems of common fixation techniques, we covered a microporous calcium phosphate carrier by a layer of tissue engineered cartilagenous tissue. Since bone will substantially integrate the bone substitute after implantation, it will result in anchorage of the cartilage-carrier construct.

We used an alternative method to generate a cartilage layer avoiding the use of synthetic scaffolds. This method was based on the ability of chondrocytes to synthesize extracellular matrix in alginate culture which stays attached to the cells after recovery from the gel. Cell associated matrix was incorporated into the constructs allowing a later formation of mechanically stable tissue with appropriate thickness for implantation into the knee joints of minipigs.

Calcibon® was chosen because of its well known properties as one of the main anorganic components of bone [10, 16]. Calcibon® was described as an ideal matrix for the growth and differentiation of osteoprogenitors *in vivo* with an excellent bone biocompatibility. Ooms et al also demonstrated that the integration into the surrounding bone starts early and that the cement surface was completely covered with newly deposited bone after 2 weeks [10]. With a compressive strength of about 50 MPa, the carrier has enough stability to hold the exposure in a human trabecular bone, where the maximum compressive strength is 30 MPa [11]. Calcium phosphate can be fabricated in any shape and porosity. It is a bioactive material with reproducible characteristics and it is degraded without the production of toxic metabolites. Moreover, cartilage will adhere to calcium phophate which was crucial for our study.

As chondrocytes dedifferentiate as early as first passage and lose their characteristic matrix synthesis [24] redifferentiation is indispensable to generate cartilagenous tissue after expansion of the cells. Yaeger et al. demonstrated a reversible dedifferentiation after transfer of cells to a suspension culture in alginate beads [19]. This method was also used in the present study.

There are only few reports on tissue engineering for the repair of deep osteochondral lesions in a large animal model [14, 15, 25]. We chose a minipig model for representative findings and to link previous experimental findings to clinical problems.Analysis was performed after 26 and 52 weeks in order to document the effects over time. Most of published studies on cartilage or osteochondral transfer and tissue engineering of cartilage were performed in rat or rabbit models.

Since the healing process of critical size osteochondral defects is well described in the literature by migration of mesenchymal cells into the damaged zone and partial reorganization into a disorganized fibrocartilage tissue that degenerates at a later stage [12–15], we decided not to perform control operations. Some authors report on the formation of subchondral cysts and host bone collapse in the trochlear groove of the minipig after 1 year [12, 13]. Several authors characterized the subchondral changes seen after implantation of auto-, allo- and xenografts in sheep and after autologous chondrocyte transplantation in goats respectively. A substantial resorption of the subchondral bone and a subchondral cyst formation were seen leading to host bone collapse, implant-dislocation and finally to cartilage matrix degradation of the grafts [26, 27]. Especially in light of these findings, one of the most favorable findings of present study is the absence of the formation of subchondral cysts.

Since the poor integration of neocartilage with recipient cartilage has been a major obstacle to articular cartilage restoration [28] some authors describe the integration of neocartilage to maintain biomechanical integrity as the ultimate success of cartilage repair [14]. In order to achieve a better healing at the cartilage interface, we cut the edges of the biphasic constructs just before the process of implantation to create a fresh surface. By this procedure a good junction and integration of the neocartilage with the native cartilage was observed *in vivo*. In contrast to the works of Lane et al. and Horas et al. who reported in long term evaluations a remaining cleft between the recipient articular cartilage and the cartilage of transferred osteochondral plugs in goats and human patients, respectively [29, 30], a complete healing of the neocartilage and no gap nor an acellular zone between the implant and the femoral condyle was observed in our study in the chondral site.

Since collagens serve important mechanical functions in the connective tissues, the development of an adequate collagen framework represents the major aim of therapeutic cartilage repair [31]. The evidence of type II collagen as major part of the extracellular matrix of hyaline cartilage is inevitable for the histological graduation of tissue engineered cartilage. In present study immunohistochemistry revealed type II collagen synthesis in all implants similar to that seen in normal adult articular cartilage. Type I collagen was also seen in all samples but was more discrete and variable than type II collagen. In some specimens on the edges of the chondral part of the implants, fibrocartilage and a mixture of fibrous and hyaline tissue could be determined.

However, the calcium phosphate carrier did not show any resorption or degradation probably due to a lacked porous architecture. Due to biomechanical forces and because of the absence of a remodeling or biodegradation of the calcium phosphate carrier some of the implants countersunk or tilted within the implantation site leaving

the surface even by increasing volume of the cartilage overlying the carrier. In consequence a partial regeneration of the subchondral bone layer growing from the sides could be observed comparable to the results of a recent study on tissue engineering for the therapy of osteochondral defects [25]. This could mean improved biomechanical properties of the implant even though it was not evaluated in this study.

The lack of reestablishing of normal subchondral bone architecture and the lack of resorption or remodeling of the calciumphospate carrier resulted certainly in inferior mechanical properties of the hyline-like tissue. So we must state, that the carrier is in need of improvement. It is tempting to speculate that the latter problem was at least in part due to the surgical technique used since exact reconstruction of osteochondral defect by *in vitro* generated biphasic construct is difficult given the variation in joint surface contour and difficult perpendicular implantation. Previous studies have shown, that a countersinking of implants is not necessarily negative. Huang et al. compared flush and countersunk osteochondral implants and saw a certain capacity for remodeling by a significant cartilage thickening; but this tolerance for incongruity was limited [32].

Even though we did not correlate radiological and MR imaging with histological findings, we can confirm, that evaluation through MR imaging provides a useful assessment of properties of the cartilage and adjacent tissue as stated by Roberts et al. [33] and Eckstein et al. [34]. It has to be mentioned, that MR imaging is not always reliable. In present study, the decreasing signal of the interface between the calcium phosphate carrier and the native bone could suggest a remodeling or an increasing ingrowth of the bone substitute into the adjacent bone. On this point, MR imaging did not correlate with histology – which did not show any remodeling of the carrier – pretending a better result due to a lacking differentiation between native bone and calcium phosphate.

In conclusion, present results demonstrate the potential benefit of an *in vitro* tissue engineered cartilage and may serve as first steps for its further development. The data are promising over all with respect to the integration of the neocartilage into the adjacent cartilage and the tissue maturation over the time. Further studies are necessary to define the redifferentiation of hyaline chondrocytes. An improvement of the calcium phosphate carrier is certainly required before this approach for the repair of osteochondral articular defects fulfills clinical expectations.

Acknowledgments The authors thank Stephanie Nagel-Heyer, Frank Feyerabend and Jens Schroeder for their technical assistance. This work was supported by the BMBF (03 N 4012).

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